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## CD99 is a novel therapeutic target for control of T-cell mediated CNS autoimmune disease

Ryan C. Winger<sup>\*,‡</sup>, Christopher T. Harp<sup>†</sup>, Ming-Yi Chiang<sup>†</sup>, David P. Sullivan<sup>\*</sup>, Richard L. Watson<sup>\*</sup>, Evan W. Weber<sup>\*</sup>, Joseph R. Podojil<sup>†</sup>, Stephen D. Miller<sup>†</sup>, and William A. Muller<sup>\*,2</sup>

<sup>\*</sup>Department of Pathology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611

<sup>†</sup>Department of Microbiology-Immunology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611

### Abstract

Leukocyte trafficking into the central nervous system (CNS) is a prominent feature driving the immunopathogenesis of multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). Blocking the recruitment of inflammatory leukocytes into the CNS represents an exploitable therapeutic target; however, the adhesion molecules that specifically regulate the step of leukocyte diapedesis into the CNS remain poorly understood. Here, we report that CD99 is critical for lymphocyte transmigration without affecting adhesion in a human blood-brain barrier model. CD99 blockade in vivo ameliorated EAE and decreased the accumulation of CNS inflammatory infiltrates, including dendritic cells, B-cells and CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. Anti-CD99 therapy was effective when administered after onset of disease symptoms and blocked relapse when administered therapeutically after disease symptoms had recurred. These findings underscore an important role for CD99 in the pathogenesis of CNS autoimmunity and suggest it may serve as a novel therapeutic target for controlling neuroinflammation.

### Keywords

CD99; Central Nervous System; Neuroinflammation; EAE; Autoimmunity; Endothelium; Blood-brain barrier; Transendothelial migration; Diapedesis; Lymphocyte; CD4<sup>+</sup> T-cell; Dendritic Cell

## INTRODUCTION

Leukocyte diapedesis (transmigration; TEM) is a tightly regulated process that is vital to inflammation and immune responses. TEM is mediated by a set of adhesive molecular interactions between circulating leukocytes (WBCs) and the vascular endothelium. During immune surveillance, a limited number of WBCs enter the CNS by crossing the blood-brain barrier (BBB) to detect potential damaging agents (1, 2); however, exacerbated WBC

<sup>2</sup>Address correspondence and reprint requests to: Dr. William A. Muller, MD, PhD, Department of Pathology, Feinberg School of Medicine, Northwestern University, 303 E. Chicago Ave., Chicago, IL 60611. wamuller@northwestern.edu, Phone:

+1-312-503-8144, Fax: +1-312-503-8249.

<sup>‡</sup>Present address: Department of Neurology, University of California San Francisco, San Francisco, CA 94143

recruitment into the CNS is a prominent pathological feature driving multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) (3, 4).

Before mediating tissue destruction in CNS autoimmunity, lymphocytes first interact with adhesion molecules on the BBB to reach their cognate Ag (5). In addition, the TEM of inflammatory APCs, including inflammatory myeloid-derived dendritic cells (iDCs), macrophages, and B-cells, from the periphery into the CNS is an early event and their accumulation persists throughout the course of disease (6–8). Therefore, elucidating the mechanisms governing the TEM of lymphocytes and inflammatory APC into the CNS is critical to understand the pathogenesis of disease. The integrin VLA-4 mediates firm adhesion to endothelium in the periphery (9–13) and the CNS (14–16) and has been demonstrated to play an integral role in inflammatory WBC accumulation in the CNS (17–20). However, inflammatory WBCs may use alternative mechanisms that specifically govern the step of diapedesis to infiltrate the CNS and mediate disease.

CD99 is a 32-kD surface glycoprotein that is concentrated at cell borders of all endothelium and is expressed diffusely on the surface of WBCs (21, 22). Homophilic WBC-endothelial CD99 interactions are critical for TEM in vitro (21–23) and in inflammatory settings within the periphery in vivo (24–26). Blocking WBC CD99 inhibits TEM to the same extent as blocking endothelial CD99 (21, 22, 24), making it an attractive therapy for inflammatory mediated diseases. Recently, we reported that CD99 mediates monocyte TEM across human BBB models in vitro (23). However, the role of CD99 for other WBC subsets at the BBB may be different and its role in the pathogenesis of CNS disease is unknown.

In the current report, we examined the requirement of CD99 for lymphocyte TEM in the BBB model in vitro and investigated the mechanism and efficacy of anti-CD99 therapy to regulate relapsing-remitting EAE (RR-EAE). RR-EAE in the SJL/J strain shares similar clinical and histopathological hallmarks that are germane to MS (27), and thus serves as a powerful tool for studying the immunopathogenesis of disease and potential therapeutic interventions (28). We show that CD99 is required for lymphocyte diapedesis but not adhesion in a human BBB model in vitro. More important, anti-CD99 therapy reduced the clinical severity of RR-EAE and diminished T-cell, iDC and B-cell accumulation in the CNS. Thus, CD99 is a novel therapeutic target for the management of CNS inflammation and autoimmunity.

## MATERIALS AND METHODS

All procedures involving human subjects/materials were approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine.

### Antibodies and reagents

Mouse anti-human mAbs for VE-cadherin (clone hec1) and CD99 (clone hec2), Armenian hamster anti-mouse PECAM (clone 2H8) and rat anti-mouse CD99 (clone 3F11) mAbs were generated by hybridoma methodologies as previously described (21, 24, 29–31). Rat anti-mouse CD3 (clone 17A2) mAb was purchased from eBioscience.

### Isolation and culture of human endothelial cells for the BBB model

The BBB model was cultured as described previously (23). Prior to TEM experiments, monolayers were activated by TNF (20ng/ml; R&D Systems) for 24h and preincubated with apical CXCL12 (200ng/ml; R&D Systems) for 20–30 min as described previously (32–34).

### Isolation of human leukocytes

Lymphocytes were isolated from human PBMC and mitogen activated (1µg/ml PHA; Sigma Aldrich and 20 ng/ml IL-2; R&D Systems) as previously described (33, 34). CD4+ and CD8+ T-cells were isolated from human PBMC by viable-cell sorting using mAb against CD3, CD4 and CD8 by the Robert H. Lurie Comprehensive Cancer Center Flow Cytometry Core Facility.

### Transendothelial migration assay

Quantitative endpoint TEM assays were performed as previously described (21, 23, 24, 32–34).

### Mice

Female SJL/J (Jackson Laboratories; Bar harbor, ME); were housed under specific pathogen-free conditions in the Northwestern University Center for Comparative Medicine Animal Facility. Northwestern University Animal Care and Use Committee approved all protocols.

### Active induction and clinical evaluation of PLP<sub>139–151</sub> mediated RR-EAE

6–8 week-old female SJL/J were immunized s.c. over three sites on the flank with 100 µl of an emulsion containing 50 µg proteolipid protein (PLP)<sub>139–151</sub> and CFA as previously described (7, 8). Disease was scored on a 0 to 5 scale, as previously described (8, 35).

### In vivo antibody treatment

RR-EAE mice were randomized at the onset of clinical symptoms and treated i.p. with 100 µg of rat-anti-CD99 mAb (clone 3F11) or control-IgG (rat IgG; JacksonImmuno Research Laboratories) beginning either at onset of symptoms or at relapse and every other day thereafter.

### In situ whole mount immunostaining

Whole mount immunostaining was performed, acquired, and analyzed as previously described (24, 36). Spinal cords (SC) were harvested intact at disease peak and relapse by flushing the vertebral column with saline (37), then fixed in 4% paraformaldehyde overnight at 4°C. This method removes the meninges. SC were blocked and permeabilized in a solution of 0.2% TritonX-100, 2.5% BSA, 2% FBS and 2.5% host-species serum in PBS overnight at 4°C. SC were incubated overnight at 4°C with primary mAb against PECAM-1 and CD3 in blocking solution. SC were incubated with DyLight 550 goat anti-Armenian hamster and DyLight 488 goat anti-rat secondary antibody in PBS at room temperature for 4h. SCs were mounted in sagittal orientation and parenchymal postcapillary venules (identified by PECAM and 20–40 µm in diameter) in the lumbar region associated with

perivascular lymphocyte infiltrates (identified by CD3) were imaged using an Ultraview Vox imaging system equipped with a Yokogawa CSU-1 spinning disk. Images were acquired using Volocity software (Perkin Elmer), which renders the optical sections into 3D images. Image processing and analysis was performed with ImageJ. The figure shows representative projections of the whole stack using the maximum intensity method.

### Cell isolation

WBCs were isolated from digested spinal cord and brain of individual PBS perfused mice via Percoll gradient as described previously (8, 38).

### FACS

CNS cells were stained with the indicated antibodies (eBioscience and BD) as described previously (8, 38, 39): CD45, CD39, CD11b, CD11c, Ly6c, CD19, plasmacytoid dendritic cell antigen (PDCA)-1, F4/80, CD3, CD4, CD8 and CD25. Intracellular staining for FoxP3 was performed per manufacturer's instructions with eBioscience staining buffer kit. Aqua/VID Live-dead cell stain was purchased from Life Technologies.

### Statistical analysis

All statistical analyses were done using GraphPad Prism software (GraphPad, San Diego, CA).

## RESULTS and DISCUSSION

### CD99 blockade restricts lymphocyte TEM but not adhesion at the BBB

Studies in EAE have demonstrated that CNS infiltrating lymphocytes play a critical role in CNS autoimmune disease. We investigated the role of CD99 for lymphocyte TEM at the inflamed BBB model in vitro. BBB model monolayers were activated as described in the *Materials and Methods*. TEM assays with polyclonal activated lymphocytes were performed in the presence of blocking mAb against CD99 or non-blocking control mAb against VE-cadherin. Under control conditions 65% of adherent lymphocytes transmigrated, while anti-CD99 blocked TEM down to ~19% (>70% block in TEM) (Fig 1A). Anti-CD99 blocked TEM of both CD4+ and CD8+ T-cells to a similar extent (Fig 1C). Under all of the conditions examined, none of the mAb treatments significantly affected lymphocyte adhesion to the monolayers (Fig 1B,D), demonstrating that our observations are specifically due to a block in TEM, as observed for monocytes (21, 23, 24) and neutrophils (22, 24, 36). This is in contrast to ICAM-1 (40), VCAM-1 (17), ALCAM (41) and Ninjurin-1 (42), which have been demonstrated to play a role in both adhesion and subsequent diapedesis of inflammatory WBCs subsets across the BBB.

### CD99 reduces clinical severity and histopathological burden of RR-EAE

To date, the role of CD99 in EAE is unknown. We determined the role of CD99 for WBC recruitment to the CNS in vivo using PLP<sub>139-151</sub> induced RR-EAE in SJL/J mice by administering anti-CD99 or control IgG i.p. once mice became symptomatic. Anti-CD99 mAb given i.p. was able to reach the CNS as it labeled endothelial junctions of the BBB (data not shown). Mice receiving anti-CD99 mAb displayed significantly lower clinical

scores than controls ( $p = 0.001$ ) at both peak disease and relapse (Fig 2A and Table I). The majority of anti-CD99 treated mice displayed mild disease and were even protected against the development of relapse (Fig 2A and Table II). Whole mount immunostaining revealed reduced histopathological burden of CD3+ T-cells in the SC of anti-CD99 treated mice compared to controls (Fig 2B). Qualitatively similar results were seen in SC of mice examined at the peak of relapse (not shown).

### **Anti-CD99 treatment diminishes inflammatory leukocyte infiltration in the CNS of RR-EAE mice**

The *in vitro* findings from Fig 1 and whole mount staining in Fig 2 suggests that the therapeutic efficacy of anti-CD99 was due to its ability to block the recruitment of lymphocytes from entering the CNS. However, similar to MS, RR-EAE is also characterized by extensive CNS perivascular infiltration of inflammatory APCs that perpetuate damage (3, 8, 43). In particular, iDCs are major players in CNS autoimmune pathology (8, 44), and their numbers correlate with disease severity and progression (5, 8, 42, 43, 45–48). The molecular mechanisms governing the TEM of this pathogenic subset into the CNS are poorly understood.

The frequency of CNS infiltrating WBC subsets known to play a role in the pathogenesis of disease that express CD99 was characterized by FACs on cells from dissociated brains and spinal cords harvested at disease peak (Figure 3A–B) from 5 individual mice. We next enumerated the different CNS infiltrating inflammatory WBC populations in anti-CD99 or control mice by performing FACS at disease peak and relapse. There was a significant reduction in the absolute numbers of CNS CD45<sup>hi</sup>-infiltrating B-cells, iDC, CD4+ and CD8+ T cells at disease peak and relapse in CD99 treated mice (Fig 3C–F). CD3+CD4+ Th1 and Th17 cells were both reduced to an equal extent in anti-CD99 treated mice (data not shown). There were no significant differences in the absolute numbers of microglia (CD45<sup>lo</sup> CD11b<sup>lo</sup> CD11c+), macrophages or plasmacytoid DCs between anti-CD99 and control IgG treated RR-EAE mice. In contrast to the CNS, we observed no significant differences in absolute cell numbers in spleen and lymph nodes between anti-CD99 treated mice and controls at any time point assessed (data not shown). In addition, at disease onset and remission there were no significant differences in cell numbers between anti-CD99 and control in any of the tissues examined. Importantly, peripheral blood WBC counts were slightly higher in CD99 treated animals compared to control IgG (Figure 3G–H). Taken together, these data suggest that anti-CD99 therapy specifically inhibited WBC migration into the CNS rather than depleting WBCs or inhibiting emigration from bone marrow.

### **Anti-CD99 blocks disease relapse in ongoing EAE and reduces leukocyte accumulation in the CNS**

In the clinic most patients with MS receive treatment following disease exacerbations, rather than prophylactically. Therefore, we took a more clinically relevant approach by treating mice with established EAE only during the secondary phase of disease at relapse onset (~23 DPI). Anti-CD99 therapy ameliorated the development and severity of the secondary phase of disease (Fig 4A). Similar to our observations in Fig 3, CD45<sup>hi</sup> CNS infiltrating iDCs, B-cells, and CD4+ and CD8+ T-cells were all significantly reduced in mice that received anti-

CD99 therapy at the onset of relapse compared to controls. Importantly, as observed in Figure 3B,D there was no reduction of CD3+CD4+CD25+Foxp3+ T-regulatory cell accumulation in the CNS following treatment with anti-CD99 (Fig 4C). Selective inhibition of effector but not regulatory T cell TEM by anti-CD99 has important implications for translation into the clinical setting.

Blocking WBC extravasation into the CNS is an exploitable and effective therapeutic target for treating CNS-directed autoimmune disease. Anti-VLA-4 (Natalizumab) is a highly potent and efficacious therapy for the management of MS. Anti-VLA-4 prevents the binding of leukocyte  $\alpha 4$  integrin to its endothelial ligand VCAM-1, thereby inhibiting adhesion and subsequent TEM into the CNS. The S1P receptor modulator FTY720 (Fingolimod) sequesters WBCs in the lungs and secondary lymphoid organs (49), thus impeding homing of WBC to the CNS. In the present report, our data demonstrate that targeting CD99 interrupts leukocyte extravasation into the CNS. Anti-CD99 specifically disrupts the step of diapedesis but not adhesion or trafficking, making it a unique therapeutic target. Unlike the steps preceding TEM in the inflammatory response, diapedesis is arguably the only committed, non-reversible step of inflammation (50). Once WBCs undergo TEM, they are committed to carrying out their effector function in the inflammatory response (51).

Preventing WBC infiltration into the CNS reduces the extent of CNS inflammation and thereby ameliorates MS symptoms; however this does not come without risk (49, 52). Both anti-VLA-4 and Fingolimod therapies have been reported to impede the ability to protect against latent and chronic CNS viral infections by impairing immune surveillance (53–56). It is unknown whether anti-CD99 therapy would increase the risk of opportunistic infection, but fortunately recent advances in the field have made it possible to assess the likelihood with simple antibody tests (49).

Taken together, our report reveals a novel role for CD99 in the TEM of inflammatory WBCs across the BBB in vivo and further emphasizes the importance of WBC recruitment in the pathogenesis of CNS autoimmune disease. Furthermore, because anti-CD99 specifically blocks the step of diapedesis, anti-CD99 or small molecule antagonists of CD99 signaling (24) might serve as exploitable therapeutic targets for suppressing ongoing neuroinflammation in CNS autoimmune disease.

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## Abbreviations used in this article

<b>TEM</b>	transendothelial migration
<b>BBB</b>	blood-brain barrier
<b>iDC</b>	inflammatory myeloid-derived dendritic cell

<b>RR-EAE</b>	relapsing remitting-experimental autoimmune encephalomyelitis
<b>SC</b>	spinal cord

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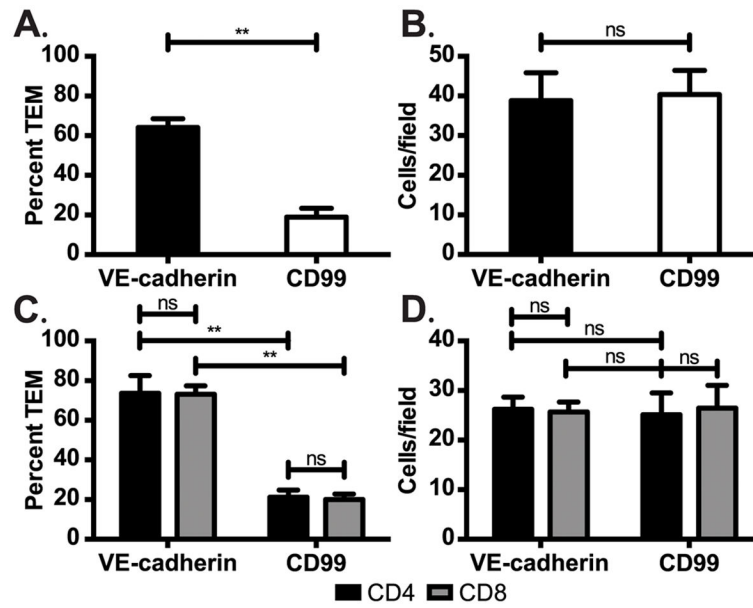
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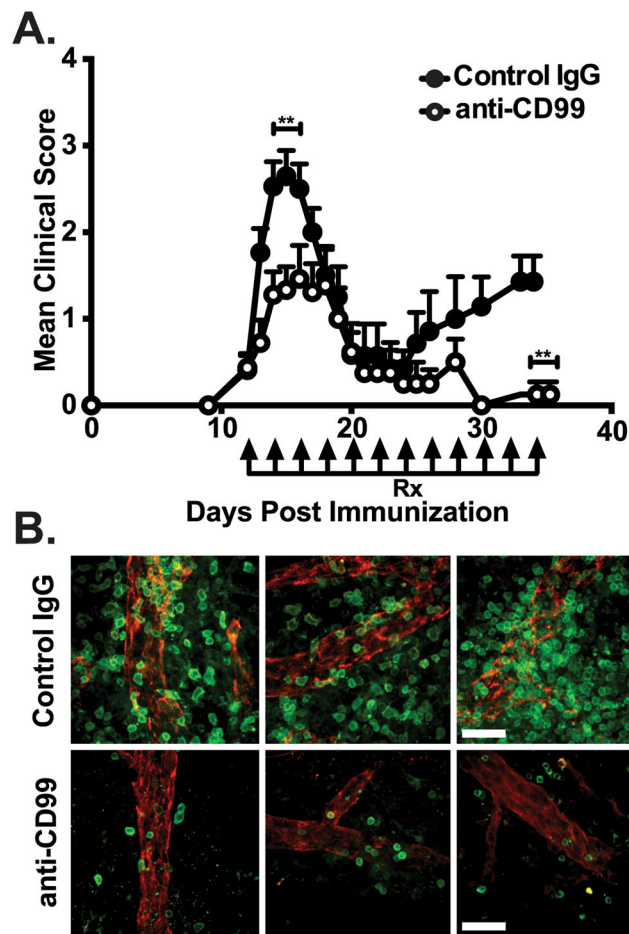


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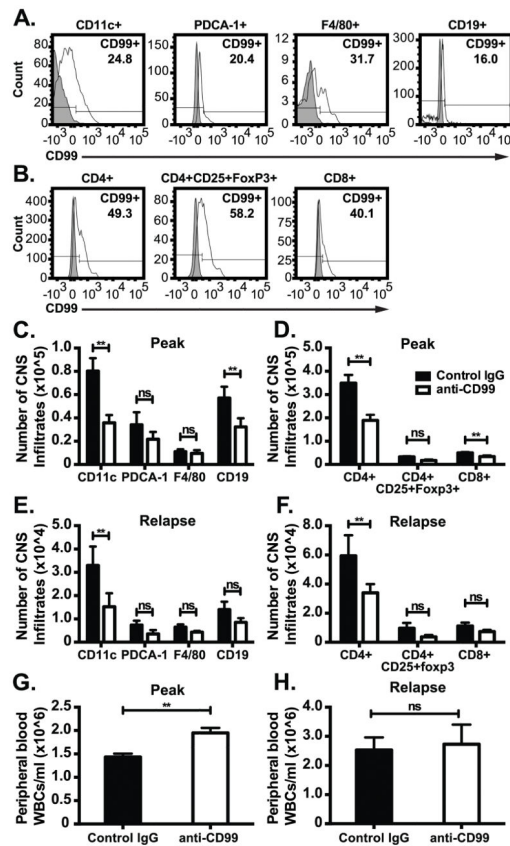
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**Figure 1. CD99 is required for lymphocyte TEM at the human BBB model in vitro**  
 TEM assays were performed with total lymphocytes (A) or purified CD4+ and CD8+ T-cells (C) on inflamed BBB model monolayers in the presence of anti-CD99 or control mAbs (non-blocking anti-VE-cadherin). (B,D) The number of lymphocytes that adhered to the monolayer were scored for multiple high power fields in each monolayer. Data represent the mean ± SD of hundreds of TEM events from at least six replicates from two (C,D) and three (A,B) independent experiments. \*\*p < 0.001 (unpaired t-Test and two-way ANOVA).

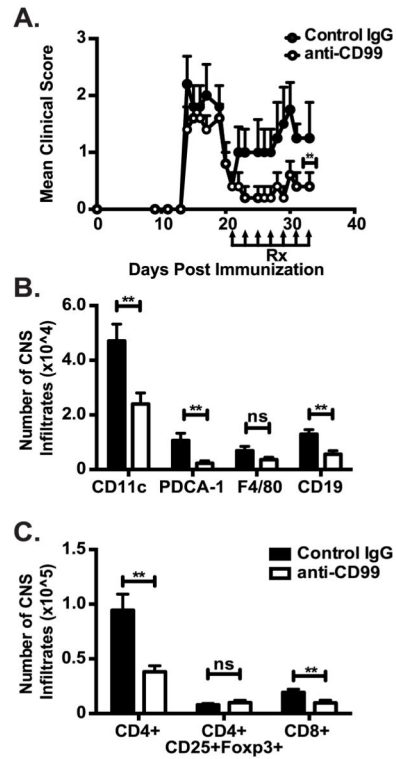


**Figure 2. Anti-CD99 ameliorates RR-EAE and reduces histopathological burden**  
**(A)** Comparison of mean clinical score between mice treated (arrows) with anti-CD99 (open circles) or control IgG (closed circles) at onset of clinical symptoms and every other day thereafter. **(B)** Whole mount immunostaining of spinal cord parenchymal postcapillary venules (PECAM-1; red) and CD3+ T-cell (green) infiltrates from anti-CD99 (lower panels) or control-IgG (upper panels) treated mice at peak of disease. Data shown are representative of 3 independent experiments **(A,B)** with a minimum of 5 mice per group and from >20 immunostainings performed on post-mortem material from 3 animals/group **(B)**. Scale bar = 26 $\mu$ m. Error bars indicate SEM. \*\* $p < 0.001$  (Mann-Whitney U test).



**Figure 3. Inhibiting CD99 function prevents accumulation of inflammatory infiltrates in the CNS of RR-EAE mice**

(A) The percentage of CD45<sup>hi</sup> infiltrating iDCs (CD11b+**CD11c**+Ly6c<sup>hi</sup>), macrophages (CD11b+CD11c-**F4/80**+), plasmacytoid DCs (CD11b-CD11c+**PDCA-1**+) and B-cells (**CD19**+), and (B) T-helper cells (CD3+**CD4**+), cytotoxic T-cells (CD3+**CD8**+) and T-regulatory cells (CD3+**CD4**+**CD25**+**Foxp3**+) expressing CD99 from the brain and spinal cord of individual mice at disease peak was quantitated by FACS. Histograms are representative of 5 individual mice. Filled bar indicates fluorescence minus one control and open line represents CD99 expressing cells. (C–F). The number of CD45<sup>hi</sup> infiltrating **CD11c**+ iDCs, **F4/80**+ macrophages, **PDCA-1**+ plasmacytoid DCs and **CD19**+ B-cells, and **CD4**+ T cells, **CD8**+ T-cells and **CD4**+**CD25**+**Foxp3**+ T-regulatory cells at disease peak (C–D) and relapse (E–F) were enumerated from brain and spinal cords of individual anti-CD99 (open bars) or control IgG (closed bars) treated mice by FACS. Bolded antigens denote respective labeling on the bar graphs. Data shown in C–F are mean absolute number of CD45<sup>hi</sup> CNS infiltrating cells pooled from 3 independent experiments obtained from at least 12 mice/group. Error bars indicate standard error of the mean. \*\*p < 0.05 by two-way ANOVA. (G–H) Peripheral blood leukocytes were from anti-CD99 or control IgG treated RR-EAE mice were collected via cardiac puncture at disease peak and relapse and manually counted (from Fig 2A). Error bars indicate SEM. \*\*p < 0.05; ns= not significant by t-Test.



**Figure 4. Anti-CD99 ameliorates relapse in ongoing RR-EAE and reduces CNS inflammatory infiltrates**

(A) Animals with ongoing RR-EAE were treated with anti-CD99 (open circles) or control IgG (closed circles) during the secondary phase of disease at the onset of relapse.  $**p < 0.001$  by Mann-Whitney U test. (B–C) CNS tissues were harvested at the end of the experiment to enumerate inflammatory APC (B) and T-cell (C) infiltrates in the CNS of RR-EAE mice as described in Fig 3. Data in A are representative of 3 independent experiments ( $n = 8$  mice/group for each experiment;  $**p < 0.001$  by Mann-Whitney U test). Data in B–C are pooled from 12-mice/treatment group. Error bars indicate SEM.  $**p < 0.05$  compared to control IgG by two way ANOVA (B,C).

**Table I**  
**Clinical scores of RR-EAE mice**

The mean clinical scores of anti-CD99 or control IgG treated mice from three independent experiments were quantitated. Mean  $\pm$  SEM.

Treatment	Onset	Peak	Remission	Relapse
Control IgG	0.34 $\pm$ 0.12	2.50 $\pm$ 0.24	0.71 $\pm$ 0.22	1.50 $\pm$ 0.31
Anti-CD99	0.36 $\pm$ 0.12 <sup>ns</sup>	1.39 $\pm$ 0.21 <sup>**</sup>	0.61 $\pm$ 0.24 <sup>ns</sup>	0.46 $\pm$ 0.14 <sup>**</sup>

ns= non-significant

<sup>\*\*</sup> p 0.001 compared to control IgG by Mann-Whitney U test

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**Table II****EAE severity of RR-EAE mice**

The percentages of mice receiving anti-CD99 or control IgG with no clinical score, mild (score 1–2) or severe disease (score 3–5).

<i>Peak</i>			
<b>Treatment</b>	<b>No clinical score</b>	<b>Mild (1–2)</b>	<b>Severe (3–5)</b>
<b>Control IgG</b>	10% (4/40)	35% (14/40)	55% (22/40)
<b>Anti-CD99</b>	25% (10/40)	62.5% (25/40)	12.5% (5/40)

<i>Relapse</i>			
<b>Treatment</b>	<b>No clinical score</b>	<b>Mild (1–2)</b>	<b>Severe (3–5)</b>
<b>Control IgG</b>	11% (2/18)	83% (15/18)	6% (1/18)
<b>Anti-CD99</b>	50% (9/18)	50% (9/18)	0% (0/18)